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The long range goal of our research is to identify genes involved in the early stages of breast cancer development, specifically genes involved in the transition from hyperplasia to ductal carcinoma in-situ (DCIS) and DCIS to invasive breast cancer. We are investigating the expression of LAF-4, a gene we found to be transcriptionally perturbed in invasive breast cancer. We have selected cases of pre-malignant breast lesions and microdissected these specimens. We developed an RT-PCR assay to evaluate LAF-4 expression during progression. Quantitative RT-PCR analysis of LAF-4 mRNA level was evaluated in ANN tumor tissue in comparison to corresponding normal mammary tissue and found to be up-regulated in 2/7 cases. We have constructed riboprobes against LAF-4 and conducted several hybridisation experiments. By studying expression of LAF-4 in a spectrum of breast lesions, it may be possible to identify whether this gene plays a role in the progression to malignancy and, in the future, to develop targeted approaches to breast cancer prevention.

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FOREWORD

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Irene L. Gabrilovich July 26, 2000

PI - Signature Date

Table of Contents

Introduction

Advances in prevention and treatment of breast cancer require an understanding of the etiology and pathogenesis of the disease. It has emerged that the evolution towards malignancy is driven by a progressive accumulation of genetic alterations. In effect, these alterations compromise the physiological activities of proto-oncogenes and tumor suppressor genes (TSGs). To date, attention has focused on relatively few genes in breast cancer. Cumulatively these genes account for only a small proportion of cases of sporadic breast cancer so it follows that many important genes are yet to be identified. The long range goal of our research is to identify genes involved in the early stages of breast cancer development, specifically genes involved in the transition from hyperplasia to ductal carcinoma in-situ (DCIS) and DCIS to invasive breast cancer. By studying these transitions it will be possible to identify key genes in the progression to malignancy and develop targeted approaches to breast cancer prevention. Our objective is to characterise the role of LAF-4 in the development of breast cancer. LAF-4 is a gene which we have recently identified as being transcriptionally perturbed in breast cancer and thus may play an important role in breast cancer development.

HYPOTHESIS/PURPOSE

The hypothesis of this work is that alterations in gene expression can compromise the physiological functions of the gene products in the cell, and thereby perturb the balance in growth and development, leading to tumorigenesis. In particular, the induction of LAF-4 may have an important role in the pathogenesis of breast cancer.

BODY

We are characterizing the functions of this gene to clarify its role in breast cancer.

Task 1 Tissue microdissection to collect a panel of samples of various types (hyperplasia, DCIS, invasive carcinoma, etc.)

We have begun to collect the cases for the study. Dr. Done, an anatomic pathologist has performed histological evaluation and selection of suitable areas for microdissection. These cases have been microdissected and RNA has been prepared.

a. Tissue microdissection

Molecular analysis of gene expression of solid tumors is largely based on mRNA analysis of crushed frozen tumor samples. Since most tumors are composed of a mixture of neoplastic cells together with inflammatory, stromal, endothelial and other cell types, molecular alterations acquired by neoplastic cells may be masked. Many methods of RNA extraction require large amounts of starting material which are not available from the lesions we will study. We have adapted a previously described method to allow it to be used for frozen tissue sections. Samples are microdissected from cryostat sections placed on 2% agarose coated glass slides. We have chosen to use cryostat rather than FFPE sections to minimize RNA degradation. Microdissected tissue is immersed in a freezing solution and rapidly freeze-thawed to lyse the cells. Aliquots can then be used directly in RT-PCR reactions without further purification. We have examined the effect of different tissue thickness and different tissue staining dyes. We estimate that a small microdissected region, containing no more than 200 cells can contain enough RNA for 80-100 RT-PCR reactions.

Task 2 Quantitative RT-PCR on microdissected samples

Given the limitation in material when working with tumors, we have used a quantitative RT-PCR approach towards evaluation of gene expression. For the evaluation of LAF-4 mRNA expression in breast tumors, tumor RNA is reverse transcribed into cDNA to serve as template in multiplex PCR reactions containing primers specific for LAF-4 as well as the house keeping gene ß2m. The relative level of LAF-4 expression is

indicated by the ratio of intensities of LAF-4 to the internal control ß2m PCR products on polyacrylamide gel stained with ethidium bromide. All quantitation is performed by laser densitometry. For each tumor sample, PCR will is performed at several different cycles of amplification to ensure that quantitation within the linear phase of PCR kinetics. We have designed primers and optimized the conditions for RT-PCR for LAF-4. We are confirming the appropriate reference gene for control and are selecting cell lines to be used as controls. To evaluate whether expression of LAF-4 is altered in breast cancer, quantitative RT-PCR analysis of LAF-4 mRNA level was evaluated in ANN tumor tissue in comparison to corresponding normal mammary tissue. It was found to be up-regulated in 2/7 cases. Analysis of LAF-4 expression in a large cohort of ANN breast tumors showed that for the majority of tumors, the distribution of LAF-4 mRNA level was comparable to that found in normal mammary tissues. However, in 11/45 ANN tumors, the level of LAF-4 mRNA was significantly higher than the highest level found in the normal tissues.

In the next year we will perform analysis of microdissected samples.

Task 3 RNA in-situ hybridization

We have constructed riboprobes against LAF-4 and conducted several hybridisation experiments. These were not of sufficient specificity and we are reconsidering this approach.

Task 4 Analysis of methylation status of promoter region

These experiments are scheduled for the next year of the grant.

We will determine the mechanism underlying LAF-4 transcriptional activation in some breast tumors, with a specific focus on the status of DNA methylation. Since the LAF-4 sequence between the transcriptional start site and the first codon is GC rich, we hypothesize that the loss of DNA methylation may explain the presence of

LAF-4 mRNA in some tumors. This will be tested using a combination of restriction enzymes that are methylation-sensitive, and PCR amplification.

KEY RESEARCH ACCOMPLISHMENTS

- Selection and microdissection of cases of pre-malignant breast lesions, in situ carcinomas and invasive breast cancers.
- Design of primers for RT-PCR and analysis of LAF-4 expression in invasive breast cancers
- In situ hybridisation probes constructed

REPORTABLE OUTCOMES

Abstracts and presentations, published or in press:

1. Done S.J., To M.D., Redston M. and Andrulis I.L. LAF-4 a Putative Proto-Oncogene ins Overexpressed in Human Breast Cancer. Proceedings of the American Association for Cancer Research (2000) 41: 442

Presentations:

1. *LAF-4 a Putative Proto-Oncogene is Overexpressed in Human Breast Cancer.* Presented at the American Association for Cancer Research Annual Meeting, San Fransisco, April 2000.
2. *Overexpression of LAF-4, a Putative Proto-oncogene, in Breast Cancer.* Presented at the Reasons for Hope Breast Cancer Research Conference, Toronto, June 1999.

CONCLUSIONS

The objectives are to characterize the functions LAF-4 to determine how perturbations in its expression contribute to tumorigenesis. Our preliminary results clearly demonstrate potential involvement in breast cancer pathogenesis of a gene, LAF-4, isolated from the dd-PCR application. We are characterizing the functions of this gene to clarify its role in breast cancer. If this gene proves to have a significant role in breast cancer, it will

be useful to evaluate its prognostic potential in ANN breast cancer in long term studies. A clear understanding of the timing of transcriptional deregulation will allow the design of targeted prevention strategies and offers the potential of substantially reducing the number of new breast cancer cases in the future.